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Separation of formoterol enantiomers and detection of zeptomolar amounts by capillary electrophoresis using laser-induced fluorescence

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Abstract

A sensitive and rapid high-performance capillary electrophoresis (HPCE) method combined with laser-induced fluorescence (LIF) detection is described, which is suitable for the analysis of racemic formoterol and formoterol enantiomers after derivatization with fluorescein isothiocyanate (FITC). The limit of detection is 1 pg/ml in the case of FITC-derivatized racemic formoterol in the absence of chiral selector. Upon injection of 5 nl, it corresponds to an amount of not more than 120 molecules. The chiral recognition occurs in the presence of heptakis(2,3,6-tri-O-methyl-β-cyclodextrin) as a chiral selector in the buffer electrolyte and yields a detection limit of $10 \cdot 10^{-12}$ g/ml for each enantiomer.

1. Introduction

Most of the chiral drugs available on the market are administered as racemates [1]. However, the great difference in pharmacological effects and pharmacokinetics between the two enantiomeric forms of many drugs is also well known [2,3]. Therefore, the pharmaceutical industry increasingly needs new analytical and preparative procedures capable of resolving and quantitating drug enantiomers, and the resolution of racemic mixtures is becoming a highly challenging area of separation technology.

High-performance capillary electrophoresis (HPCE) is a relatively new mode of analytical separation with great potential and is already applied to a wide variety of molecules, ranging from simple ions to larger particles, and for ionized as well as neutral compounds [4]. This technique has already attracted a considerable amount of attention in different areas such as analytical biochemistry, molecular biology, analytical chemistry, and medical biology. One particularly important application of HPCE is the chiral separation, where the technique offers new alternatives to the already existing methods. This application has already been the subject of different reviews [5–9]. Cyclodextrins and their derivatives are the most commonly used chiral selectors in HPCE due to their capacity to include a wide range of compounds and the generally high degree of chiral recognition they afford. We have already shown that HPCE is a very suitable enantioselective analytical tool for resolving chiral pharmaceuticals in terms of high speed, high resolving power, short optimization

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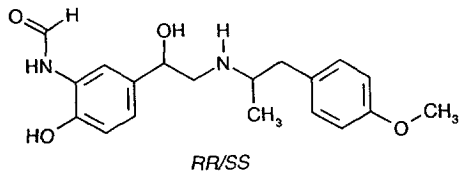


RR/SS

Fig. 1. Chemical structure of racemic formoterol (*RR/SS*).

time, and low cost [10]. Formoterol (Fig. 1), a relatively new and extremely potent oral β2-adrenoreceptor agonist with a long-lasting bronchodilator action, is administrated as a racemate. The very low dosage required (usually 15 μg per inhalation) results in low plasma concentrations (generally <50 pg/ml). Consequently, the development of an enantioselective analytical method allowing the separation and the detection of the enantiomer at very low concentrations constituted a real challenge.

In this work, we describe two rapid and sensitive analytical methods using HPCE in combination with laser-induced fluorescence detection (1) for the determination of racemic formoterol that has been derivatized with FITC and (2) for the enantiomeric separation of FITC-derivatized formoterol.

2. Experimental

2.1. Chemicals

All the cyclodextrins used, α-CD, β-CD, γ-CD, heptakis(2,6-di-O-methyl-β-CD) and heptakis(2,3,6-tri-O-methyl-β-CD) were purchased from Sigma Chemical (Buchs, Switzerland). Fluorescein isothiocyanate isomer I (FITC) and 1-octane sulfonic acid sodium salt monohydrate were from Fluka Chemicals (Buchs, Switzerland). The racemic formoterol was from Ciba Pharma (Basel, Switzerland). All other reagents and solvents were of analytical grade. It should be pointed out that special demands must be placed on the purity of the solvents used in fluorescence measurements since considerable quenching can be caused by impurities.

2.2. Formoterol derivatization

A solution of 0.1285 g (0.33 m*M*) of fluorescein isothiocyanate dissolved in 15 ml of absolute ethanol is added dropwise and at room temperature to a mixture of 0.103 g (0.3 m*M*) of racemic formoterol and 0.5 ml of triethylamine dissolved in 15 ml of absolute ethanol. After addition, the mixture is stirred for 4 h at 40°C. The solvent is evaporated and the residue is purified by chromatography on silica gel (eluent: chloroform–ethylacetate–methanol, 4:6:6). The fractions containing the desired compound are collected and yield, after evaporation, 188 mg of the pure formoterol derivative (yield 85.5%). The NMR, elemental analysis, and mass spectroscopy data are in accordance with the expected structure.

2.3. Instrumentation

The experiments were carried out on a Beckman PACE 2100 capillary electrophoresis system equipped with a LIF detector and a power supply capable of delivering up to 30 kV. The excitation light from a 4-mW argon-ion laser was focused on the capillary window by means of a fiber-optic connection. Excitation was performed at 488 nm and a 520-nm band-pass filter was used for emission. A fused-silica capillary (Supelco, Gland, Switzerland), with 75 μm I.D., 57 cm total length, and 50 cm from the point of sample introduction to the detector window, was used in all the experiments. Data acquisition was performed using the Gold chromatography software package system.

Injections were made using the pressure mode (ca. 0.3 psi) for 5 s each. In all the experiments a constant voltage was applied and the temperature of the separation system was kept at 25°C during the run. In all cases the migration direction was toward the cathode.

The fluorescence measurements for determination of excitation and emission maxima of the FITC-derivatized formoterol were performed with a Perkin-Elmer MPF 66 spectrofluorimeter.

2.4. Running conditions

For the analysis of FITC-derivatized formoterol, the following mixed buffer was used: 10 m*M* borate buffer (pH 9) with 10% methanol. For the enantiomeric resolution of the FITC-derivatized formoterol, a buffer mixture of 10% methanol, 67 m*M* phosphate buffer (pH 8) was prepared and the appropriate amount of cyclodextrin was added. Prior to analysis, the capillary was rinsed with 0.1 *M* NaOH for 2 min and then filled with the electrophoretic buffer. All the buffer solutions were filtered through a membrane filter (Skan, Basel, Switzerland) of 0.2-μm pore size prior to use. Stock solution of FITC-derivatized formoterol for LIF detection was prepared in methanol and diluted 10^9 times by steps of 10.

3. Results and discussion

Formoterol is administrated as a racemic mixture of both *RR*- and *SS*-enantiomers. Various methods were previously developed to detect the racemic drug, and the lowest limit of detection (20 pg/ml) has recently been obtained using HPLC and electrochemical detection with a signal-to-noise ratio of 3:1 [11]. However, in a recent report it has been shown that the therapeutic activity of formoterol probably resides only in the *RR*-enantiomer [12]. The enantioselective analysis of the samples was performed on a Chiral-AGP column using UV detection [13]. But, owing to the low plasma concentration, a more sensitive and reproducible analytical method is required to determine the enantiomeric composition of samples to evaluate the pharmacokinetic profile of both enantiomers.

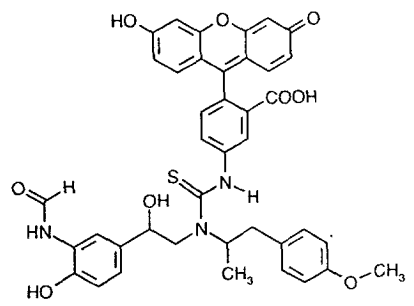
HPCE with γ-cyclodextrin as a chiral selector has already been used as a means to analyze the enantiomeric composition of formoterol samples [14]. This chiral separation was achieved using a phosphate buffer (pH 3) containing 40 m*M* γ-cyclodextrin and 40 m*M* of the sodium salt of 1-octane sulfonic acid monohydrate. However, the on-line UV detection is not capable of attaining the sensitivity needed to perform studies in the desired concentration range (ca. 10 pg/ml). This limitation is essentially due to the short path-length provided by the capillary tubing.

To date, the most sensitive detector for capillary electrophoresis is based on laser-induced fluorescence (LIF) [15] and it has already been used for various applications [16,17], including enantiomeric separations [18,19]. For example, with suitable tags, mass sensitivities on the zeptomolar level (10^{-21} moles or hundreds of molecules) have been obtained for amino acids by using capillary laser-induced fluorescence [20,21]. Unfortunately, the fundamental limitation of fluorescence detection is that relatively few molecules fluoresce. Then, most of the analytes have to be derivatized (pre- or post-column derivatization) with a fluorescence reagent.

3.1. Formoterol derivatization

To ensure the detection of fluorescence, formoterol has to be derivatized. This derivatization has been accomplished using fluorescein isothiocyanate isomer I. Isothiocyanates are known to react selectively with the amino group of amino alcohols and of amino phenols [22]. It consists of pre-column off-line derivatization; the reaction is carried out prior to injection of the sample into the capillary. This procedure does not impose any restrictions on the CE system. Reaction of racemic formoterol with 1.1 equivalent of fluorescein isothiocyanate in the presence of triethylamine in ethanol affords the desired derivative (Fig. 2), which has been subsequently purified by chromatography on silica gel. The reaction occurs regioselectively on the secondary amine, as indicated by the strong displacement observed in NMR spectroscopy for the protons of the $-CH_2NCH$ group, which are shifted respectively of about 2.7 ppm (CH) and 1 ppm (CH_2) to lower field. The derivative is very stable and can be stored as a stock solution for several weeks in methanol without observing any decomposition.

As can be seen from Fig. 3, the emission spectrum shows a maximum at wavelength 519



RR/SS

Fig. 2. Chemical structure of FITC-derivatized formoterol (*RR/SS*).

nm following excitation at 488 nm. The excitation spectrum gives a maximum at 499 nm with emission at 520 nm. This observed excitation maximum conveniently matches the argon-ion laser 488 nm line, and the emitted light can be measured near 520 nm.

3.2. FITC-derivatized formoterol analysis (achiral conditions)

As already mentioned above, chiral separation of formoterol using CE has been carried out under acidic conditions (pH 3) [14]. But, as formoterol has to be derivatized to enhance detection, we had to develop a new separation scheme for FITC-derivatized formoterol. It must be noted that each molecule of FITC coupled to formoterol incorporates a negative charge into the adduct, due to the presence of the carboxylic group on the label.

After optimization of the buffer conditions, a 10 m*M* borate buffer (pH 9) containing 10% methanol as organic modifier was selected for the analysis of the FITC-derivatized formoterol. Under those alkaline conditions, the electroosmotic flow is high enough to permit the elution of the compound within 7 min. The presence of 10% methanol in the running buffer contributes to band sharpness. The theoretical plate value is about 200 000 per m, which demonstrates the high resolving power of the method. The electropherogram (Fig. 4) shows that a concentration of 1 pg/ml of racemic FITC-derivatized formoterol is still detectable with a signal-to-noise ratio of about 10.

3.3. Chiral separation

To obtain chiral separation of the enantiomers of racemic FITC-derivatized formoterol, we evaluated different cyclodextrins and cyclodextrin (CD) derivatives as possible chiral selectors. Among the different cyclodextrins used for this

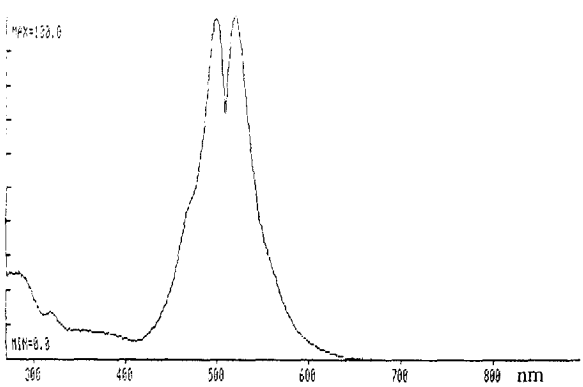


Fig. 3. Fluorescence spectra of FITC-derivatized formoterol.

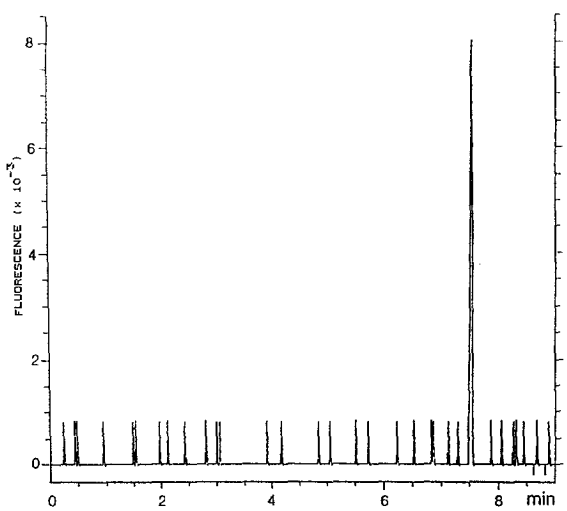


Fig. 4. Analysis of racemic FITC-derivatized formoterol. Sample: 1 pg/ml injected by the pressure mode for 5 s (ca. 5 nl). The electrophoresis was performed in pH 9 borate buffer containing 10% methanol. Applied voltage: 20 kV; LIF detection (argon-ion laser, excitation: 488 nm; emission: 520 nm).

study, α-CD, β-CD, γ-CD, heptakis(2,6-di-O-methyl-β-CD) and heptakis(2,3,6-tri-O-methyl-β-CD), only the latter compound was effective in achieving optical resolution of the FITC-derivatized formoterol. The type of CD as well as the structural features of the molecule play an important role in resolution. The β-CD derivative consists of seven glucopyranose units in which the three OH groups in positions 2, 3, and 6 of every unit are converted into methoxyl groups.

The effects of buffer pH, methanol percentage, and CD concentration on the enantiomeric resolution of FITC-derivatized formoterol were investigated to determine the optimum separation conditions. According to the results obtained above, our investigations were made at alkaline pH 7–9. In this pH range, the resolution improves as the pH of the running buffer decreases. Therefore, we select pH 8, which affords a good compromise for good resolution, short migration time, and high sensitivity.

We also studied the influence of the percentage of methanol used as an organic modifier. The methanol content in the electrophoretic buffer was first increased by adding 10% methanol. At higher percentages of methanol, no further improvement in resolution was observed, and the analysis time was dramatically lengthened due to the decrease in electroosmotic velocity (Table 1).

The influence of concentration of the added heptakis(2,3,6-tri-O-methyl-β-CD) (TM-β-CD) in the running buffer on the resolution (R_s) was also investigated. The resolution increases rapidly from 5 m*M* TM-β-CD ($R_s = 1.02$) to 20 m*M* TM-β-CD ($R_s = 1.12$). Higher CD concentrations do not markedly further improve the resolution and have the disadvantage of increasing the retention time of the enantiomers.

Table 1
Influence of methanol percentage on the enantiomeric resolution

Methanol (vol%)	Migration time (min)	Resolution R_s
0	14.32	1.16
10	19.70	1.26
20	26.55	1.22

Buffer electrolyte: phosphate buffer, pH 8, containing 20 m*M* TM-β-CD and different amounts of methanol. Applied voltage: 10 kV.

A phosphate buffer (pH 8) containing 10% methanol and 20 m*M* TM-β-CD was the most appropriate to achieve both high enantiomeric resolution and high sensitivity. At a concentration of 10 pg/ml, the enantiomers are still well detectable (Fig. 5a). The very low detection limits obtained in this study are essentially due to the use of a suitable fluorogenic label, FITC isomer I, that is compatible with the spectral characteristics of the argon-ion laser.

We also investigated the possibility of enantiomeric resolution in the micellar electrokinetic capillary chromatography (MECC) mode. As already mentioned, separation of formoterol enantiomers with on-line UV detection has been achieved under acidic conditions and in the presence of the sodium salt of 1-octane sulfonic acid monohydrate. By adding 40 m*M* octane sulfonic acid Na salt to the buffer, FITC-derivatized formoterol was optically resolved as shown in Fig. 5b. The result is almost similar to that obtained in the capillary zone electrophoresis (CZE) mode. The migration time is slightly longer, which is certainly due to the presence of the micelles.

The MECC mode has some advantages over the CZE mode, because it is possible to modulate the migration time and the capacity factor by modifying both the CD and the micellar concentration, resulting in easier optimization of the separation. In addition, we have already demonstrated the possibility of directly determining hexobarbital enantiomers in rat plasma using CD-MECC [10]. In this case, the plasma proteins were solubilized by the micelle [sodium dodecyl sulfate (SDS)], and the migration times were selectively manipulated to avoid any interference with drug enantiomers. CD-MECC has the merit of allowing direct and rapid analysis of drug enantiomers in biological samples without any pretreatment such as deproteinization or extraction.

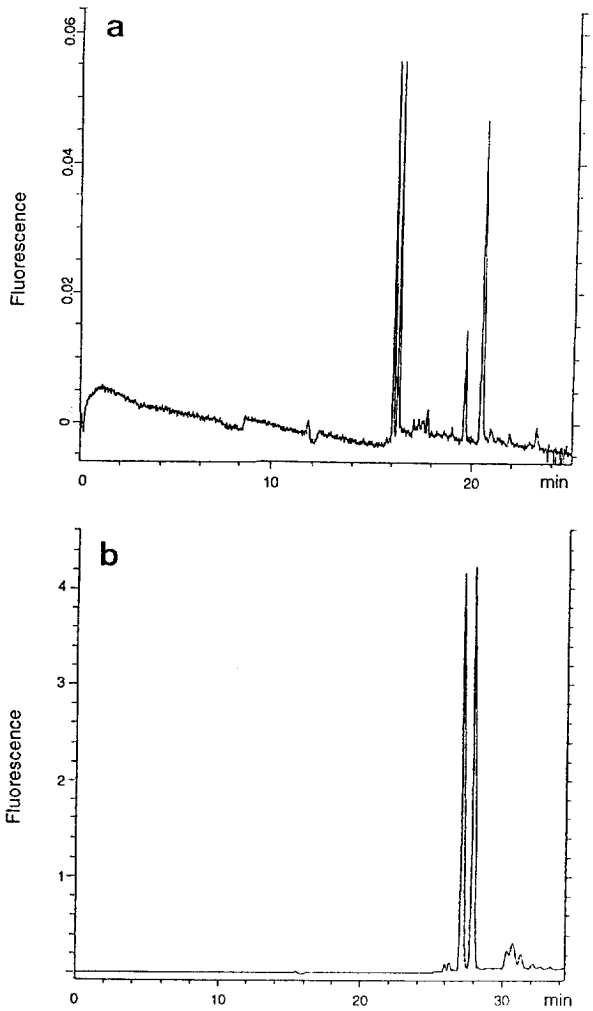


Fig. 5. Enantiomeric resolution of FITC-derivatized formoterol. LIF detection (argon-ion laser, excitation: 488 nm; emission: 520 nm). Injection by the pressure mode for 5 s (ca. 5 nl). (a) CZE resolution: sample, 10 pg/ml; buffer electrolyte, phosphate buffer (pH 8) containing 20 m*M* TM-β-CD and 10% methanol. Applied voltage: 12 kV. (b) MECC resolution: sample, 0.1 ng/ml; buffer electrolyte, phosphate buffer (pH 8) containing 20 m*M* TM-β-CD, 40 m*M* octane sulfonic acid, and 10% methanol. Applied voltage: 11 kV.

4. Conclusion

The combination of capillary electrophoresis with laser-induced fluorescence detection permits the analysis of samples containing very low concentrations of analytes. This technique is particularly suitable for highly potent drugs active at extremely low doses, such as formoterol, a relatively new oral β2-adrenoreceptor agonist. The high sensitivity of the method has been clearly demonstrated by the reported application to formoterol, which, after derivatization with fluorescein isothiocyanate, could be detected at a concentration of $1 \cdot 10^{-12}$ g/ml. Using an appropriate chiral selector in the buffer electrolyte, it is possible to simultaneously separate the enantiomers of formoterol detected in the $10 \cdot 10^{-12}$ g/ml concentration range. This application to the separation and detection of the enantiomers of a chiral drug at such a low concentration demonstrates the promising potential of the technique.

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